Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: https://www.tandfonline.com/loi/ienz20

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To cite this article: Francesca Margheri, Mariangela Ceruso, Fabrizio Carta, Anna Laurenzana, Laura Maggi, Simone Lazzeri, Gabriele Simonini, Francesco Annunziato, Mario Del Rosso, Claudiu T. Supuran & Rolando Cimaz (2016) Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium, Journal of Enzyme Inhibition and Medicinal Chemistry, 31:sup4, 60-63, DOI: 10.1080/14756366.2016.1217857

To link to this article: https://doi.org/10.1080/14756366.2016.1217857

Journal of Enzyme Inhibition and Medicinal Chemistry

www.tandfonline.com/ienz
ISSN: 1475-6366 (print), 1475-6374 (electronic)

J Enzyme Inhib Med Chem, 2016; 31(S4): 60–63
 DOI: 10.1080/14756366.2016.1217857

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RESEARCH ARTICLE

Overexpression of the transmembrane carbonic anhydrase isoforms IX and XII in the inflamed synovium

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Abstract

Juvenile idiopathic arthritis (JIA) is the most common form of chronic rheumatic disease affecting children worldwide, with some features similar to adult rheumatoid arthritis (RA). In the present study, we aim at investigating novel markers that will allow in the future for tailored, more personalized treatment strategies. Hence, taking notice of several reports proving the role of local acidosis as a causal link between inflammatory diseases and related pain, and the involvement of several carbonic anhydrases (CA, EC 4.2.1.1) isoforms in articular diseases, we evaluated in JIA patients the expression of these metalloenzymes. We identified that JIA patients show high levels of active CA IX and XII isoforms. Our results represent the first evidence of the identification of these enzymes as potential therapeutic targets and development of novel innovative therapies for arthritis, also considering that the two isoforms are validated antitumor targets.

Keywords

Carbonic anhydrase, juvenile idiopathic arthritis, synovium

History

Received 12 July 2016
Accepted 25 July 2016
Published online 12 August 2016

Introduction

The synovial membrane may become inflamed and thickened in conditions such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA), the latter been the most common chronic rheumatic disease in the pediatric age. The pathophysiology of JIA has been related to that of RA, which is considered a chronic autoimmune disorder. Inflammatory cells such as T lymphocytes produce cytokines that sustain the process of synovial pannus-driven extracellular proteolysis and proliferation, and promote the inflammation-associated angiogenesis. Very old observations report high hydrogen ions concentration in inflamed tissues and have highlighted the importance of low pH of the synovial fluid of RA patients, thus indicating that pH becomes more acidic as the intensity of the inflammatory reaction increases[1]. Local acidosis has been suggested to provide a causal link between inflammatory diseases and their related pain[2]. Data also indirectly point to low-pH-dependent reduction in the proliferation of lymphocytes and polymorphs, impaired chemotaxis and inhibition of antibody production[3].

Several forms of carbonic anhydrases (CAs, EC 4.2.1.1), metalloenzymes that reversibly catalyze the conversion of CO_2 to the bicarbonate ions and protons[4], have been recognized in articular diseases, including CA I which is over-expressed in the synovium of the patients with ankylosing spondylitis[5]. Transgenic mice over-expressing CA I showed aggravated joint inflammation and destruction[6]. CA I has been identified as an oral biomarker in oral fluid of Sjogren's syndrome[7], and antibodies to CA III and IV have been identified in rheumatoid arthritis[8]. In consideration of the paucity of data on CAs in rheumatic diseases, we have studied the presence of CA IX and XII, two emerging CAs which are pivotal in many human pathologies[4], in the synovial fluid and synovial cells of JIA patients. Our results show a significant increase of CA IX and CA XII protein and activity in biological specimens from JIA patients.

Materials and methods

Sample preparation

Whole blood samples from healthy donors and from the patients with JIA were collected by using EDTA, heparin and acid citrate dextrose (ACD) as anticoagulants after informed consent and with local Ethics Committee approval. The tubes were processed immediately, or after overnight storage at 4 °C. Samples were prepared after diluting a 3 to 5 ml aliquot of blood 1:2 with Millipore water (milliQ), mixed by vortex for 3 min and stored at 4 °C for 24 h. The samples were thus centrifuged for 30 min at 6000 rpm at 4 °C and the pellet was discarded. Samples of synovial fluid, previously taken during therapeutic arthrocentesis from patients with JIA, were stored at −18 °C until analysis (minimum an overnight). After thaw at room temperature, samples were analyzed with the same protocol.

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Synovial samples

Synovial fibroblasts were derived from 7 JIA patients (mean age: 9 years, range: 9–15 years) and 6 healthy controls (mean age: 8 years, range: 5–10 years), after informed written consent and with the approval of Ethics Committee of Anna Meyer Children Hospital, Florence, Italy.

For patients under the age of 18 years, written consent was obtained by the parents or guardians. All the procedures of this study have been conducted according to the principles expressed in the Declaration of Helsinki.

Healthy synovial tissue was removed from knee joints of controls during orthopedic surgery for traumatic fractures or elective procedures, minced into small pieces and plated in culture dishes with FBM-2 (Fibroblast Growth Medium, Microtech, Napoli, Italy) supplemented with 10% FBS for expansion. JIA synovial fibroblasts were obtained from synovial fluid of JIA patients, as previously described[9].

CA concentration determination

The protein concentration in the samples was determined spectrophotometrically by measuring the absorbance at 280 nm using a molar extinction coefficient $\varepsilon = 50\,070\ M^{-1}\,cm^{-1}$ calculated using A280 (1%) of 19.0 according to Nyman and Lindskog protocol[10] and a molecular weight value of 30 kDa for CA II, 59 kDA for CA IX and 54 kDA for CA XII (dimeric enzymes)[11,12]. Absorption spectrophotometry was carried out using a Cary 50 Conc UV/Visible spectrophotometer connected to a Lauda A100 thermostatic water bath to maintain a constant temperature in the cell holder.

CA IX and XII inhibition assay

The presence of active CA in the enzyme solutions freshly made up from the lysed biological samples was determined by using a stopped-flow assay. 0.1 μM solution of the enzyme was obtained by diluting determined amount of the blood/serum/synovial fluid/synoviocytes samples (based on the protein content previously determined by A_{280}) in a buffer containing 10 mM Hepes (pH 7.4), 10 mM Tris.HCl and 0.1 M $NaSO_4$. Phenol red (at a concentration of 0.2 mM) was added to the enzyme solutions and used as pH indicator. An applied photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity (Leatherhead, Surrey, UK)[13]. Phenol red has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.4), 10 mM Tris. HCl and 0.1 M $NaSO_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the catalytic activity and inhibition constants. The pan-inhibitor acetazolamide (**AAZ**) and the CA IX/XII-selective Coumarin inhibitor **C** were used to assess the inhibition of various CA isoforms. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM for **AAZ** or **C**) were prepared in distilled-deionized water and dilutions up to 100 or 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were pre-incubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3. The kinetic parameters for the uninhibited enzymes were derived from Lineweaver–Burk plots, as reported earlier[4,14,15], and represent the mean from at least three different determinations.

Quantitative real-time PCR

Total RNA was prepared using Nucleospin RNA II (Macherey-Nagel, Carlo Erba Reagents, Milano, Italy), agarose gel was checked for integrity, and reverse transcribed with GoScript system (Promega, Madison, WI) using random primers according to manufacturer's instructions. CA IX and CA XII expression in normal SFs under various conditions was determined by a quantitative Real-Time (RT)–PCR with an Applied Biosystem 7500 Fast Real Time PCR System (Applied Biosystems, Carlsbad, CA) and determined by the comparative Ct method using 18S ribosomal RNA as the normalization gene. Amplification was performed with the default PCR setting: 40 cycles of 95 °C for 15 s and of 60 °C for 60 seconds using SYBR Green–based detection (GoTaq qPCR Master Mix; Promega, Madison, WI). Primers (IDT, TemaRicerca) used for RT-PCR were as follows:

18S-rRNA sense 5′-CCAGTAAGTGCGGGTCATAAG-3′, antisense 5′-GCCTCACATA-ACCATCCAATC-3′; CAIX sense 5′-GGAAGGCTCAGAGACTCA-3′, antisense 5′-CTTAG-CACTCCAGCAGTCAC-3′; CAXII sense 5′-CTGCCAGCAAC AAGTCCAG-3′, antisense 5′-ATATTCAGCGGTCCTCTC-3′.

Western blotting

Aliquots of lysates (30 μg) of normal and JIA synovial fibroblasts were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. Membranes were probed with primary antibodies against: CAIX and CAXII (Sigma-Aldrich, St. Louis, MO), while tubulin (Sigma-Aldrich, St. Louis, MO) was used as loading control. Suitable peroxidase-conjugated IgG preparations (Sigma-Aldrich, St. Louis, MO) have been used as secondary antibodies; the ECL procedure was employed for development.

Results and discussion

The total CA activity was measured in whole blood, serum, synovial fluid (only in patients) and synoviocytes from healthy donors ($n = 5$) and JIA patients ($n = 14$) by using a CO_2hydrase stopped-flow technique (Table 1). The pan-CA inhibitor AAZ as well as the isoform-selective Coumarin inhibitor **C** (which does not inhibit the cytosolic, red blood cell isoforms CAI and II, but selectively inhibits in the low nM range the transmembrane isoforms CA IX and XII) were also used in these experiments in

Table 1. Levels of carbonic anhydrase present in the lysed samples of blood, serum, synovial fluid and synoviocytes (from healthy controls or JIA patients) and inhibition data with the standard sulfonamide inhibitor acetazolamide (**AAZ**) and CA IX/CA XII-selective Coumarin inhibitor **C** (Figure 1) by a stopped flow CO_2 hydrase assay.

Sample	% CA activity	
	Healthy donors	JIA patients
Blood	100 ± 3.9	100 ± 6.2
Serum	100 ± 5.1	100 ± 4.8
Synovial fluid	NO	135.4 ± 12
Synoviocytes	100 ± 4.7	129.2 ± 8.1
Blood + **AAZ**, 10 μM	6.8 ± 0.2	5.9 ± 0.5
Serum + **AAZ**, 10 μM	6.3 ± 0.5	6.1 ± 0.4
Synovial fluid + **AAZ**, 10 μM	NO	5.5 ± 0.2
Synoviocytes + **AAZ**, 10 μM	6.0 ± 4.2	5.9 ± 0.7
Blood + Coumarin **C**, 10 μM	100 ± 5.1	100 ± 4.5
Serum + **C**, 10 μM	100 ± 3.9	100 ± 4.5
Synovial fluid + **C**, 10 μM	NO	25.8 ± 3.4
Synoviocytes + **C**, 10 μM	16.0 ± 33.8	23.4 ± 2.3

order to try to recognize the contribute of various CA isoforms to the measured activity.

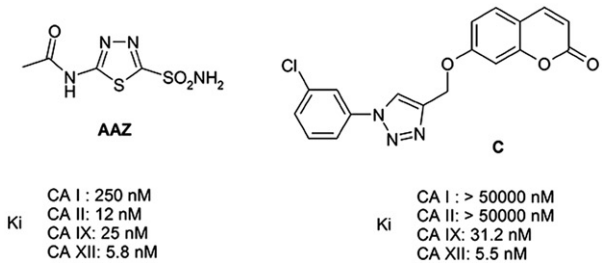

Figure 1. The pan-inhibitor of CAs acetazolamide (**AAZ**) and its inhibition constants against isoforms CA I, II, IX and XII, and the newly developed, isoform-selective Coumarin inhibitor **C**, and its inhibitory activity against the same isoforms.

As seen from the data of Table 1, the CA activity in blood and serum was identical (within the limits of the experimental error) in the samples from healthy subjects and JIA patients, whereas the synovioctyes had an increased total CA activity of 129.2%, in patients compared controls. We have also investigated the response of the samples to inhibition with **AAZ** (a compound inhibiting all CA isoforms) and Coumarin **C**, which is a CA IX/XII selective inhibitor (not inhibiting the cytosolic CA I and II, Figure 1).

As seen in Table 1, **AAZ** (at 10 μM) totally inhibited the CA activity in all samples, as expected, since this compound has a good affinity for all CAs, the widespread (in blood) isoforms CA I and II, as well as for transmembrane isoforms (which are normally absent from blood/plasma). There were no significant differences between patients and controls in their response to **AAZ** inhibition.

Coumarin **C** on the other hand showed no inhibitory effects against blood and serum samples (both in healthy subjects and in patients), as expected, since there should be no transmembrane CA isoforms in these fluids. However, a strong inhibitory effect of

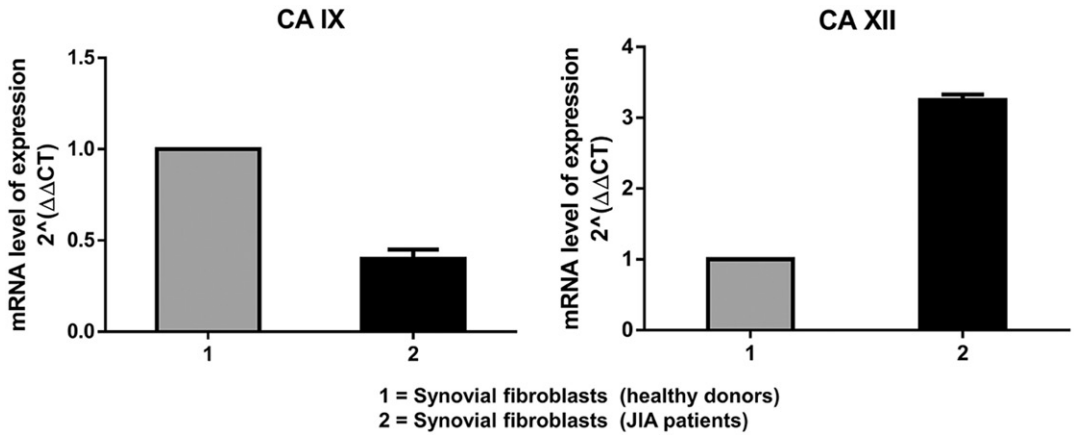

Figure 2. The CA IX and CA XII mRNA expression levels were quantified in healthy and JIA synovial fibroblasts using real time-PCR. 18s was used as housekeeping gene. Results of three independent experiments performed in triplicates are expressed as fold change according to $2^{-\Delta\Delta CT}$ method.

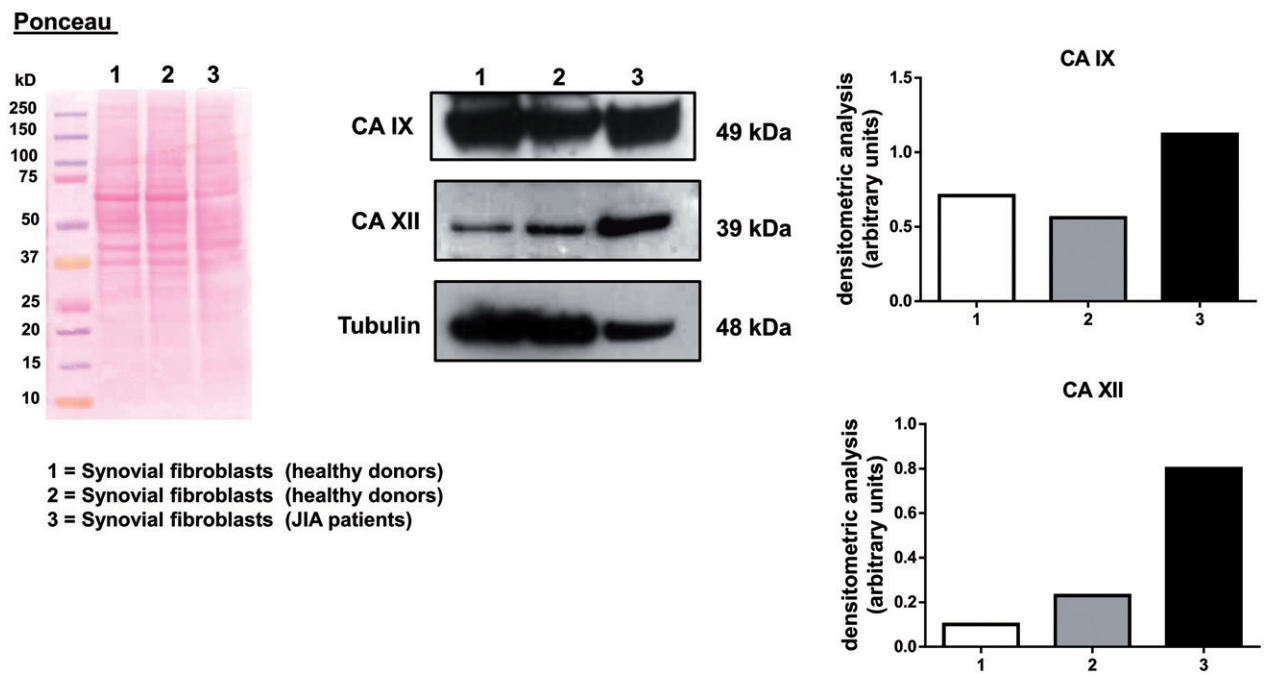

Figure 3. Western blotting detection of CA IX and CA XII in synovial fibroblasts from healthy donors and from JIA patients. Tubulin expression and Ponceau staining are used as a control for protein loading. Histograms on the right report band densitometry.

the CA activity from the synovial fluid, and from synoviocytes of both healthy subjects and patients has been observed, which is indicative of the presence of CA IX and XII in SF and synoviocytes. This is the first report for the presence of isoforms normally associated with tumors in these tissues/fluids. It should be however mentioned that both CA IX and XII are also found in some normal tissues, such as body lining and stomach (CA IX) and kidneys, brain and ciliary processes of the eye (CA XII).

To investigate whether the increased activity of CA IX and CA XII was due to an increase of the two enzymes at mRNA and protein levels, real time-PCR and Western blot analysis were performed (Figures 2 and 3) on healthy and JIA synovial fibroblasts. The data show that JIA fibroblasts displayed higher CA XII mRNA levels compared with synovial fibroblasts from healthy donors, while CA IX expression was found surprisingly higher in the latter. However, Western blot analysis showed an increase of both CA XII (8-fold higher) and CA IX protein levels in JIA synovial fibroblasts. This may be due to some hypoxia that is probably present in these compartments, and as well known, hypoxia triggers over-expression of CA IX (and sometimes also CA XII) through the hypoxia-inducible factor 1α (HIF-1α) pathway[4].

To our knowledge this is the first report on CA IX and XII overexpression in the inflamed synovium. Indeed, these findings may have a translational relevance, since beside the current use of CA inhibitors for the treatment of various pathologies such as tumors, obesity, epilepsy, they may be of critical importance for the development of novel therapeutic strategies for arthritis.

Declaration of interest

The authors state no conflict of interest.

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